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Cultural conditions on the production of extracellular enzymes by *Trichoderma* isolates from tobacco rhizosphere

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ABSTRACT

Twelve isolates of *Trichoderma* spp. isolated from tobacco rhizosphere were evaluated for their ability to produce chitinase and β-1,3-glucanase extracellular hydrolytic enzymes. Isolates ThJt1 and TvHt2, out of 12 isolates, produced maximum activities of chitinase and β-1,3-glucanase, respectively. *In vitro* production of chitinase and β-1,3-glucanase by isolates ThJt1 and TvHt2 was tested under different cultural conditions. The enzyme activities were significantly influenced by acidic pH and the optimum temperature was 30 °C. The chitin and cell walls of *Sclerotium rolfsii*, as carbon sources, supported the maximum and significantly higher chitinase activity by both isolates. The chitinase activity of isolate ThJt1 was suppressed significantly by fructose (80.28%), followed by glucose (77.42%), whereas the β-1,3-glucanase activity of ThJt1 and both enzymes of isolate TvHt2 were significantly suppressed by fructose, followed by sucrose. Ammonium nitrate as nitrogen source supported the maximum activity of chitinase in both isolates, whereas urea was a poor nitrogen source. Production of both enzymes by the isolates was significantly influenced by the cultural conditions. Thus, the isolates ThJt1 and TvHt2 showed higher levels of chitinase and β-1,3-glucanase activities and were capable of hydrolyzing the mycelium of *S. rolfsii* infecting tobacco. These organisms can be used therefore for assessment of their synergism in biomass production and biocontrol efficacy and for their field biocontrol ability against *S. rolfsii* and *Pythium aphanidermatum* infecting tobacco.

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Introduction

In the recent years, biological control of plant disease, especially soil-borne plant pathogens and nematodes by microorganisms, has been considered to be a more natural and environmentally acceptable alternative to the existing chemical treatment methods.[1] The genus *Trichoderma* is the most common saprophytic fungi in the rhizosphere; it is found in almost any soil. The mycoparasitic ability of *Trichoderma* species against some economically important aerial and soil-borne plant pathogens[2] and nematodes[3] allows the development of biocontrol strategies. The biocontrol efficacy of *Trichoderma* depends largely on the physical, chemical and biological condition of soil. There have been numerous recent attempts to use *Trichoderma* spp. on soil-borne pathogens such as *Sclerotium*, *Fusarium*, *Pythium* and *Rhizoctonia* species on different crops.[2,4]

Successful biological control systems commonly employ naturally occurring, antagonistic microorganisms that can effectively reduce activities of plant pathogens.[5] Cook[6] suggested that microorganisms isolated from the root or rhizosphere of a specific crop may be better adapted to that crop and may provide better control of diseases than organisms originally isolated from other plant species. Species of the genus *Trichoderma* have a wide biotechnological interest; however, their use as biocontrol agents requires a comprehensive analysis of the biological principles of their action.[7] The antagonistic abilities of *Trichoderma* spp. are a combination of several mechanisms, including direct mycoparasitism, which involves the production of cell-wall-degrading enzymes (CWDE).[8,9] *Trichoderma* spp. are frequently associated with both biocontrol activity and promotion of plant and root growth.[10,11] Screening of diverse population of biocontrol agents is an important requirement for developing efficient biocontrol agents. Therefore, it is imperative to index biocontrol agents prevailing in the area concerned. Tobacco is one of the important quality-conscious commercial crops grown in India. The presence of fungal diseases in tobacco and its economical consequences require the use of many fungicides. *Sclerotium rolfsii* and *Pythium aphanidermatum* cause serious diseases in tobacco nurseries, leading to death of seedlings. There is no resistance to *S. rolfsii* and *P. aphanidermatum* in the available cultivars. The objective of this study was to investigate the effect of *in vitro* cultural conditions on the production of chitinase and β-1,3-glucanase enzymes by 2 out of 12 selected *Trichoderma* isolates collected from tobacco rhizosphere from different regions.

Materials and methods

Source of organisms

Trichoderma isolates were obtained from the Division of Plant Protection, CTRI, Rajahmundry, and their places of isolation were given in Table 1. The 12 isolates were maintained on potato dextrose agar at 25 ± 2 °C.

Table 1 – *Trichoderma* isolates and their places of isolation.

S. No.	Isolate	Source of isolate
1	ThRt1	*T. harzianum*, Rajahmundry, FCV tobacco
2	ThRt2	*T. harzianum*, Rajahmundry, FCV tobacco, nursery
3	ThJT1	*T. harzianum*, Jeelugumilli, FCV tobacco
4	ThJnt	*T. harzianum*, Jeelugumilli, *Natu* tobacco
5	TvJt1	*T. viride*, Jeelugumilli, FCV tobacco
6	ThDt1	*T. harzianum*, Dinhata, WB, *mothihari* tobacco
7	ThDt2	*T. harzianum*, Dinhata, WB, *Jati* tobacco
8	ThHt1	*T. harzianum*, Hunsur, Karnataka, FCV tobacco
9	TvHt2	*T. viride*, Hunsur, Karnataka, FCV tobacco
10	ThHt3	*T. harzianum*, Hunsur, Karnataka, FCV tobacco
11	ThJO1	*T. harzianum*, Jeelugumilli, oil palm
12	Th C	*T. harzianum*, Commercial isolate

Media and cultural conditions

The *Trichoderma* isolates were grown on buffered minimal synthetic medium (MSM)[12] containing the following components (in grams per litre): $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; $FeSO_4 \cdot 7H_2O$, 0.002; $MnSO_4$, 0.002; and $ZnSO_4$, 0.002. The medium was supplemented with the appropriate carbon source (0.2%) and the pH was set to 6.0. Fifty mL of medium was dispensed into 250 mL Erlenmeyer flasks and sterilized at 1.2 kg/cm^{-1}. The flasks were inoculated (triplicate sets) with inoculum (5 mm disc) of actively growing mycelial mat from PDA plate and were incubated at 28 ± 2 °C for 6 days with shaking at 110 rpm for 12 h in a day. Culture filtrates were separated from mycelial mat by filtering through Whatman No. 1 filter paper and supernatant was centrifuged at 4 °C for 15 min at 15,000 rpm and used for estimation of enzyme activity.

Preparation of *S. rolfsii* cell walls

The cell walls of *S. rolfsii* were prepared by inoculating 100 mL of PD medium in 250 mL Erlenmeyer flasks with actively growing mycelium of *S. rolfsii* disc (0.5 mm). The inoculated flasks were incubated at 25 ± 3 °C for 7 days. The mycelium was then collected by filtration through Whatman No. 1 filter paper, washed with distilled water and homogenized in liquid nitrogen and the powder was stored in −80 °C. At the time of use the mycelial powder is homogenized with distilled water and centrifuged at 6000 rpm for 10 min and the pellet was dried in oven and used as carbon source.

Estimation of enzyme activity

For estimation of chitinase and β-1,3-glucanase activities, the isolates were grown on MSM containing chitin for chitinase and laminarin for β-1,3-glucanase activity.

Effect of pH on enzyme activity

To study the effect of pH of the growth medium on the enzyme activities, the pH of media containing chitin and laminarin for activities of chitinase and β-1,3-glucanase, respectively, was adjusted with KOH and HCl to 3, 4, 5, 5.5, 6, 6.5, 7, 8 and 9.

Effect of incubation temperature on enzyme activities

To study the effect of temperature during the growth on the enzyme activities of chitinase and β-1,3-glucanase, the isolates were grown in MSM with chitin (pH 6.0) and laminarin (pH 5.8), respectively, by incubating the flasks at 20, 25, 30, 35 and 40 °C for 6 days.

Effect of carbon and nitrogen sources on enzyme activities

The isolates were grown in MSM supplemented with different carbon sources (0.2%). Chitin, cell walls of *S. rolfsii*, glucose, fructose, sucrose, starch, galactose, maltose, succinic acid and citric acid were used as carbon sources. The effect of nitrogen sources on enzyme activities was tested in MSM containing different nitrogen sources. Ammonium nitrate, sodium nitrate, sodium nitrite, ammonium sulphate, urea, calcium ammonium nitrate, glycine and glutamic acid were used as nitrogen sources along with chitin for chitinase and laminarin for β-1,3-glucanase. To study the effect of carbon sources on repression of enzyme activity, isolates were grown in MSM with 0.2% of chitin along with 0.2% of glucose, fructose, sucrose, starch, galactose, maltose, succinic acid or citric acid for chitinase activity and 0.2% laminarin along with 0.2% of carbon sources for β-1,3-glucanase as in the case of chitinase.

Assay of enzyme activities

β-1,3-glucanase (EC 3.2.1.58) activity was estimated according to the method of Elad et al.[13] The reaction mixture contained 0.5 mL culture filtrate, 1 mL citrate buffer (pH 4.5, 0.1 M) and 0.5 mL laminarin. Test tubes containing the reaction mixture were incubated at 40 °C for 1 h and kept in boiling water bath for 5 min to stop the reaction. 2 mL of dinitrosalicylic acid reagent (1% solution of dinitrosalicylic acid in 0.7 M NaOH) was added to the reaction mixture and kept in boiling water bath for 15 min. After cooling to room temperature, the absorbance of the reaction mixture was measured at 575 nm and the amount of glucose released was estimated from standard curve prepared with glucose. The enzyme activity was expressed as nkat/mL (nmol/s). Chitinase activity (EC 3.2.1.14) was estimated[12] by incubating the reaction mixture containing 0.5 mL colloidal chitin, 1 mL McIlvaine's buffer (pH 4) and 0.5 mL culture filtrate at 37 °C for 2 h. At the end of incubation, 3 mL of potassium ferricyanide reagent (0.05% potassium ferricyanide in 0.05% sodium carbonate) was added and incubated in boiling water bath for 15 min. The amount of N-acetyl glucosamine released was estimated by measuring the absorbance at 420 nm and comparing with the standard curve prepared with N-acetyl glucosamine. The activity of the enzyme was expressed in pkat/mL (pmol/s).

The data in all the above experiments were analyzed using SAS 9.3. The data were expressed as mean ± SD ($n = 3$). Different letters in each column represent significant differences ($p < 0.05$).

Table 2 – Activities of extracellular enzymes produced by different *Trichoderma* isolates.

Isolate	Chitinase (pkat/mL)	β-1,3-Glucanase (nkat/mL)
ThRt1	40.26 ± 1.96 f	4.26 ± 0.19 c
ThRt2	42.82 ± 1.49 ef	5.22 ± 0.10 c
ThJt1	62.12 ± 3.15 a	6.75 ± 0.14 b
ThJnt	40.12 ± 1.61 f	4.06 ± 0.13 c
TvJt1	52.12 ± 2.42 bc	3.86 ± 0.17 c
ThDt1	48.24 ± 1.61 cd	4.26 ± 0.07 c
ThDt2	46.14 ± 1.22 de	4.34 ± 0.27 c
ThHt1	41.34 ± 1.50 f	4.86 ± 0.25 c
TvHt2	56.24 ± 0.59 b	9.94 ± 0.19 a
ThHt3	48.26 ± 1.48 cd	5.08 ± 0.11 c
ThJO1	46.12 ± 1.41 de	4.75 ± 0.21 c
ThC	38.26 ± 1.14 f	4.48 ± 0.30 c

The different letters in the columns represent significant differences ($p < 0.05$).

Results

The production of chitinase was studied by growing the *Trichoderma* isolates in minimal synthetic medium (MSM) containing 1% colloidal chitin as carbon source. The isolate ThJt1 showed maximum and significantly higher chitinase activity compared to other isolates (Table 2). The isolate TvHt2 was the second highest producer of chitinase, with production significantly higher than those of other isolates except ThJt1. The isolates ThDt1 and ThDt2 collected from Dinhata were on a par in chitinase activity.

The production of β-1,3-glucanase by *Trichoderma* isolates was assessed by growing the isolates in MSM with laminarin (1%) as carbon source. The isolate TvHt2 showed maximum and significantly higher β-1,3-glucanase activity when compared to all other isolates (Table 2). The isolate ThJt1 showed the second highest activity of β-1,3-glucanase. All other isolates showed β-1,3-glucanase activity but they were all on a par. Isolate TvHt2 showed nearly 61.66% higher β-1,3-glucanase activity when compared to the isolate TvJt1. The isolates ThDt1 and ThDt2 from Dinhata showed similar activity of β-1,3-glucanase.

To study the effect of cultural conditions on enzyme production, the isolates ThJt1 and TvHt2 were selected based on their superiority in the production of extracellular enzymes and inhibition of *Sclerotium* and *Pythium* mycelial growth (data not shown).

Effect of pH on extracellular enzyme activities of Trichoderma isolates

Chitinase activity increased significantly with increase in pH from 3 to 6; and on further increase in pH, the activity decreased significantly in both isolates of ThJt1 and TvHt2 (Table 3). The isolate ThJt1 showed maximum chitinase activity at pH 6 (62.80 pkat/mL), which was significantly higher than those at other pH levels. The chitinase activity decreased by

Table 3 – Effect of pH on activities of chitinase and β-1,3-glucanase of *Trichoderma* isolates ThJt1 and TvHt2.

pH	ThJt1		TvHt2	
	Chitinase (pkat/mL)	β-1,3-Glucanase (nkat/mL)	Chitinase (pkat/mL)	β-1,3-Glucanase (nkat/mL)
3	12.42 ± 0.58 g	2.26 ± 0.12 f	16.41 ± 0.95 f	2.04 ± 0.12 f
4	36.60 ± 1.25 d	6.97 ± 0.29 bc	30.46 ± 0.95 d	6.40 ± 0.31 d
5	52.40 ± 1.72 bc	7.19 ± 0.45 ab	48.92 ± 1.58 a	9.14 ± 0.27 b
5.5	55.36 ± 2.14 b	7.88 ± 0.39 a	50.24 ± 1.88 a	9.88 ± 0.25 a
6	62.80 ± 2.44 a	6.26 ± 0.37 c	52.17 ± 1.91 a	8.05 ± 0.23 c
6.5	50.18 ± 2.82 c	5.04 ± 0.22 d	44.24 ± 1.82 b	8.12 ± 0.26 c
7	38.40 ± 2.08 d	3.24 ± 0.24 e	39.32 ± 1.15 c	3.46 ± 0.17 e
8	29.60 ± 0.89 e	3.09 ± 0.09 ef	24.38 ± 0.91 e	3.08 ± 0.11 e
9	20.70 ± 195 f	2.18 ± 0.11 g	15.84 ± 1.44 f	1.88 ± 0.17 f

39.24% and 67.03% with increase in pH from 6 to 7 and 6 to 9, respectively. The isolate TvHt2 showed maximum β-1,3-glucanase activity at pH 5.5. The β-1,3-glucanase activity of isolate TvHt2 increased by 79.35% with increase in pH from 3 to 5.5 and significantly decreased by increasing pH from 5.5 to 6 (Table 3). With increase in pH from 5.5 to 9, the β-1,3-glucanase activity was decreased by 80.79%. The isolate ThJt1 showed 16.92% higher chitinase activity compared to the isolate TvHt2, whereas the isolate TvHt2 showed 20.24% higher β-1,3-glucanase activity compared to the isolate ThJt1.

Effect of temperature on extracellular enzyme activities of Trichoderma isolates

The chitinase activity of both isolates increased significantly with increase in incubation temperature from 15 to 30 °C (Fig. 1). The chitinase activity of isolate ThJt1 was maximum at 30 °C but it was on a par with the activity at 25 °C and significantly higher than at 15 °C and 20 °C. With increase in temperature from 30 °C to 35 °C, the chitinase activity significantly decreased by 41.19%.

The β-1,3-glucanase activity increased significantly with increase in incubation temperature from 15 to 25 °C; and on further increase in temperature from 25 to 35 °C, there was non-significant increase in activity (Fig. 2). The activities of β-1,3-glucanase at the incubation temperatures of 25, 30 and 35 °C were on a par. The β-1,3-glucanase activity increased by 87.25% with increase in temperature from 15 to 35 °C (Fig. 2).

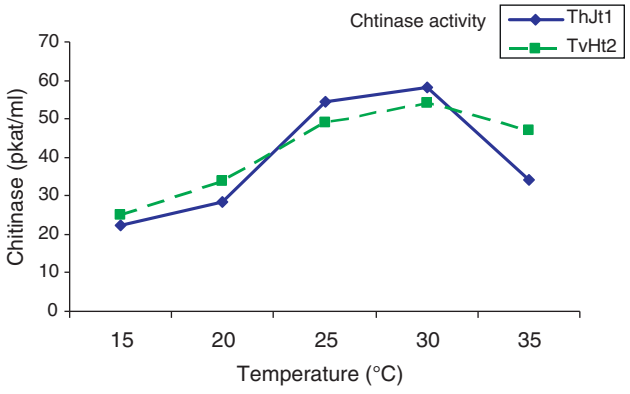

Fig. 1 – Effect of temperature on chitinase activity of *Trichoderma* isolates ThJt1 and TvHt2.

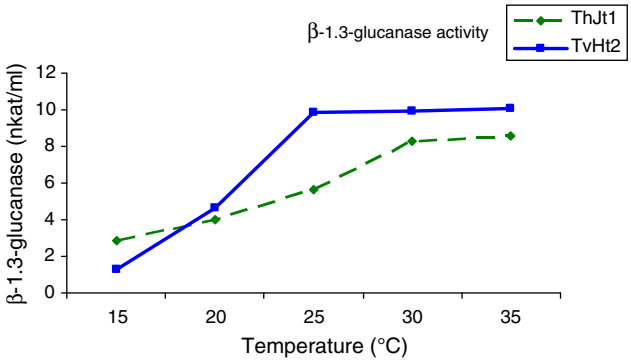

Fig. 2 – Effect of temperature on β-1,3-glucanase activity of *Trichoderma* isolates ThJt1 and TvHt2.

Effect of chitin/laminarin content on extracellular enzyme activities of Trichoderma isolates

The effect of different levels of chitin on chitinase and laminarin on β-1,3-glucanase activity was studied by incubating the isolates in MSM with different concentrations of chitin/laminarin as carbon sources. The chitinase activity of isolate ThJt1 increased significantly with increase in chitin content from 0.25% to 1% and showed maximum activity with 1% chitin (Fig. 3). The chitinase activity remained the same when the chitin content increased from 1% to 2% and the activity was decreased marginally with increase in chitin content from 2% to 2.5%. The chitinase activity increased by 53.30%

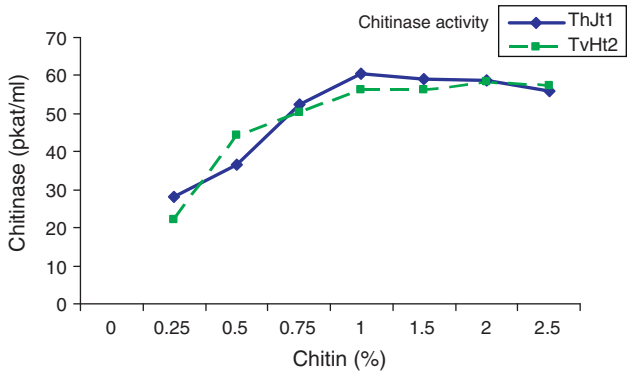

Fig. 3 – Effect of chitin concentration on chitinase activity of *Trichoderma* isolates ThJt1 and TvHt2.

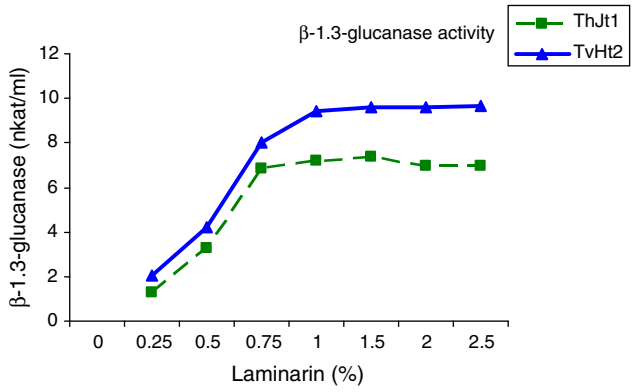

Fig. 4 – Effect of laminarin concentration on β-1,3-glucanase activity of *Trichoderma* isolates ThJt1 and TvHt2.

with increase in chitin content in the medium from 0.25% to 1%, whereas it decreased by 17.26% with increase in chitin content from 1% to 2.5%. The isolate TvHt2 showed maximum chitinase activity with 1% chitin in the medium; the activity remained the same with 1.5% and 2.5% of chitin (Fig. 4). The β-1,3-glucanase activity of the isolate TvHt2 increased with increase in laminarin content and was maximum at 1%, remaining the same with 1.5%, 2.0% and 2.5% of laminarin in the medium.

Effect of carbon sources on the extracellular enzyme activities of Trichoderma isolates

The isolate ThJt1 showed maximum chitinase activity on chitin followed by cell walls as carbon source and significantly lower activity on other carbon sources (Table 4). The lowest activity was on maltose. The chitinase activity decreased by 89.68%, 88.69% and 86.38% when maltose, fructose and sucrose were used as carbon sources, respectively, compared with the activity when using chitin. The isolate ThJt1 showed maximum β-1,3-glucanase activity on cell walls, followed by laminarin, and showed significantly lower activities on other carbon sources (Table 4). The β-1,3-glucanase activities on succinic acid- and citric acid-containing medium were on a par. Among the sugars, β-1,3-glucanase activity was maximum on the galactose and minimum on fructose. The β-1,3-glucanase activity decreased by 88.8%, 86.8% and 84.26% on fructose, sucrose and glucose, respectively, when compared with the activity when using laminarin as carbon source. The chitinase activities on glucose and on succinic acid were on a par and significantly higher than on other carbon sources. The isolate TvHt2 showed maximum β-1,3-glucanase activity on cell walls as carbon source, followed by laminarin, and showed significantly lower activity on all other carbon sources (Table 4). The β-1,3-glucanase activities on galactose, maltose, succinic acid and citric acid were on a par. The isolate TvHt2 showed the least β-1,3-glucanase activity on sucrose.

Effect of carbon sources in combination with chitin/laminarin on the extracellular enzyme activities of Trichoderma isolates

The effect of carbon sources in combination with chitin for chitinase and laminarin for β-1,3-glucanase was studied by incubating the isolates in MSM containing chitin/laminarin with different carbon sources at 1:1 ratio. The isolate ThJt1 showed maximum chitinase activity on chitin alone and the activity significantly decreased with addition of carbon sources (Table 5). The maximum suppression of chitinase activity was showed by fructose, followed by glucose. The β-1,3-glucanase activity was maximum on laminarin alone and significantly decreased with addition of other carbon sources (Table 5). The suppression of β-1,3-glucanase activity was maximum on fructose, followed by those on sucrose and starch. The β-1,3-glucanase activity was inhibited by 98.7%, 86.60% and 86.03% by fructose, sucrose and starch, respectively.

The isolate TvHt2 showed maximum chitinase activity on chitin alone and the activity decreased significantly with addition of other carbon sources (Table 5). The chitinase activity was suppressed to the maximum by fructose, followed by sucrose and galactose. The β-1,3-glucanase activity was maximum on laminarin alone and the activity decreased significantly with addition of other carbon sources (Table 5). The suppression of β-1,3-glucanase activity by the addition of other carbon sources varied from 72.96% to 87.39%.

Table 4 – Effect of carbon sources on activities of chitinase and β-1,3-glucanase of *Trichoderma* isolates ThJt1 and TvHt2.

Carbon source	ThJt1		TvHt2	
	Chitinase (pkat/mL)	β-1,3-Glucanase (n kat/mL)	Chitinase (pkat/mL)	β-1,3-Glucanase (nkat/mL)
Chitin	60.66 ± 1.80 a	7.88 ± 0.64 a	54.88 ± 2.04 a	10.29 ± 0.47 a
Cell walls	58.66 ± 0.68 b	7.94 ± 0.68 a	56.24 ± 0.77 a	10.36 ± 0.14 a
Glucose	9.46 ± 0.69 de	1.24 ± 0.12 cd	12.40 ± 0.74 bc	1.26 ± 0.05 d
Fructose	6.86 ± 0.35 f	0.88 ± 0.05 d	10.20 ± 0.44 bcd	0.98 ± 0.10 d
Sucrose	10.25 ± 0.54 d	1.04 ± 0.04 d	8.66 ± 0.91 de	0.88 ± 0.06 d
Starch	8.26 ± 0.52 e	1.88 ± 0.15 bc	7.42 ± 0.32 c	1.40 ± 0.15 cd
Galactose	8.66 ± 0.41 e	2.01 ± 0.09 b	10.24 ± 0.61 bc	1.84 ± 0.10 bc
Maltose	6.26 ± 1.38 f	1.82 ± 0.15 bc	7.48 ± 0.47 c	1.92 ± 0.13 bc
Succinic acid	12.26 ± 1.06 c	2.20 ± 0.14 b	13.24 ± 0.69 b	2.34 ± 0.13 b
Citric acid	11.06 ± 0.68 cd	2.36 ± 0.44 b	9.88 ± 0.69 d	2.08 ± 0.14 b

Table 5 – Effect of chitin + carbon sources on activities of chitinase and β-1,3-glucanase of *Trichoderma* isolates ThJt1 and TvHt2.

Carbon source	ThJt1		TvHt1	
	Chitinase (pkat/mL)	β-1,3-Glucanase (nkat/mL)	Chitinase (pkat/mL)	β-1,3-Glucanase (nkat/mL)
Chitin	62.19 ± 1.20 a	7.02 ± 0.48 a	55.53 ± 2.80 a	9.84 ± 0.37 a
Glucose	14.04 ± 0.79 e	1.83 ± 0.12 b	12.40 ± 0.77 c	1.98 ± 0.27 bc
Fructose	12.26 ± 0.46 e	0.86 ± 0.07 c	6.37 ± 0.61 d	1.24 ± 0.14 d
Sucrose	18.20 ± 0.71 cd	0.94 ± 0.14 c	8.24 ± 0.78 cd	1.28 ± 0.19 d
Starch	21.47 ± 1.55 b	0.98 ± 0.04 c	20.24 ± 1.12 b	1.62 ± 0.09 cd
Galactose	17.64 ± 1.48 d	1.98 ± 0.15 b	9.46 ± 1.11 c	2.04 ± 0.15 b
Maltose	16.48 ± 0.75 d	2.00 ± 0.14 b	18.24 ± 0.75 b	1.98 ± 0.12 bc
Succinic acid	20.23 ± 91 b c	2.20 ± 0.15 b	20.24 ± 0.95 b	2.66 ± 0.32 b
Citric acid	22.44 ± 1.31 b	1.94 ± 0.10 b	18.26 ± 1.84 b	2.14 ± 0.19 b

Effect of nitrogen sources on the extracellular enzyme activities of Trichoderma isolates

The isolate ThJt1 showed maximum and significantly higher chitinase activity on ammonium nitrate, followed by calcium ammonium nitrate (Table 6). The activity of chitinase was the least on urea. Glutamic acid showed significantly higher chitinase activity compared to glycine. The isolate ThJt1 showed maximum β-1,3-glucanase activity on ammonium nitrate, which was on a par with the activity on CAN. The β-1,3-glucanase activity was the least on urea as nitrogen source. The isolate TvHt2 showed maximum chitinase activity on ammonium nitrate, which was on a par with the activities on sodium nitrate and calcium ammonium nitrate (Table 6). The β-1,3-glucanase activities on sodium nitrate, calcium ammonium nitrate, glutamic acid and glycine were on a par. The chitinase activity was the least on urea. The β-1,3-glucanase activity of the isolate TvHt2 varied from 1.21 to 10.86 nkat/mL among the different nitrogen sources (Table 6).

Discussion

Several *Trichoderma* species reduce the incidence of soil-borne plant pathogenic fungi under natural conditions[14]; however, their efficacy depends largely on the physical, chemical and biological condition of soil. The results of this study show that *Trichoderma* isolates ThJt1 and TvHt2 produce enzymes capable of degrading chitin and β,-1,3-glucan, two major cell wall compounds of *S. rolfsii* and *P. aphanidermatum*. In the presence of chitin, laminarin and *S. rolfsii* cell wall fragments, *Trichoderma* isolates produced significant amounts of both chitinases and β-1,3-glucanases. These lytic enzymes, which are key enzymes in the lyses of cell walls of higher fungi, are produced by other organisms that are known to attack and parasitize fungi.[15] The direct mycoparasitic activity of *Trichoderma* species has been proposed as one of the major mechanisms for their antagonistic activity against phytopathogenic fungi.[16] *Trichoderma* spp. attach to the host hyphae by coiling, hooks or appressorium-like structures and penetrate the host cell walls by secreting hydrolytic enzymes such as a basic proteinase,[17] β-1,3-glucanase and chitinase.[13] Chitinase and β-1,3-glucanase enzyme production was favoured by acidic pH (6.0, 5.8). Acidic pH was also reported to be an important growth parameter in the production of chitinase and β-1,3-glucanase in *Trichoderma harzianum*.[18] Ulhoa and Peberdy[19] found that the production of chitinase was markedly affected by pH, with an optimum pH of 6.0. The pH and the optimum temperature for the production of enzymes in the present investigation were supported by the findings of Jijakli and Lepoivre[20] and Someshwar Bhagat and Sitansu Pan.[18] These results suggest that production of both chitinases and β-1,3-glucanases may be coordinately regulated since both enzymes were influenced in the same way by similar alterations of growth parameters in the culture media.

The higher activities of chitinase and β-1,3-glucanase were observed when the medium was supplemented with chitin/dried cell walls of *S. rolfsii* or laminarin; the very

Table 6 – Effect of nitrogen sources on activities of chitinase and β-1,3-glucanase of *Trichoderma* isolates ThJt1 and TvHt2.

Nitrogen source	ThJt1		TvHt2	
	Chitinase (pkat/mL)	β-1,3-Glucanase (nkat/mL)	Chitinase (pkat/mL)	β-1,3-Glucanase (nkat/mL)
Ammonium nitrate	66.28 ± 2.28 a	8.04 ± 0.58 a	56.46 ± 1.32 a	10.86 ± 0.45 a
Sodium nitrate	48.44 ± 1.21 c	6.28 ± 0.30 c	52.42 ± 1.40 abc	7.24 ± 0.30 cd
Sodium nitrite	40.26 ± 132 d	5.26 ± 0.23 d	38.46 ± 2.10 f	6.67 ± 0.22 d
Ammonium sulphate	50.48 ± 1.48 c	4.96 ± 0.24 d	44.24 ± 2.05 ef	5.06 ± 0.53 e
Urea	10.24 ± 0.90 e	1.04 ± 0.04 e	10.84 ± 0.90 g	1.21 ± 0.13 f
CAN	58.26 ± 1.04 b	7.28 ± 0.38 ab	54.91 ± 1.67 ab	8.24 ± 0.37 bc
Glycine	42.06 ± 2.15 d	6.26 ± 0.19 c	48.24 ± 1.18 cde	7.06 ± 0.18 de
Glutamic acid	48.24 ± 2.84 c	6.88 ± 0.32 bc	50.24 ± 1.39 bcd	7.16 ± 0.16 cd

low activities, with sugars and other carbon sources tested. The production of extracellular β-1,3-glucanases, chitinases and proteinase increases significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls.[9,21] The activities of chitinase and β-1,3-glucanase decreased significantly when the medium was supplemented with other carbon sources along with chitin/laminarin, indicating the repression of enzyme production by these carbon sources. No production or low production of chitinase activity in deprived carbon sources confirms that chitinase enzyme is produced inducibly and not constitutively. The expression of these cell wall-degrading enzymes has been frequently reported to be induced by fungal cell wall components and repressed by carbon catabolite repressors such as glucose and fructose.[22] The findings of El-Katatny et al.[23] were in agreement with these findings and found high chitinase activity only in cultures supplied with chitin but not with other polymers such as cellulose and chitosan, which is further indicative of induction. These observations, together with the fact that chitin, β-1,3-glucan and proteins are the main structural components of most fungal cell walls,[24] are the basis for the suggestion that hydrolytic enzymes produced by some *Trichoderma* spp. play an important role in the destruction of plant pathogens.[25] Kumar and Gupta[26] reported that cell walls of *Macrophomina phaseolina* and *S. rolfsii* are known to contain glucan and chitin, which should have resulted in the induction of chitinase and glucanase in mycelium-containing medium. *Trichoderma* spp. (especially *T. harzianum* and *T. viride*) exhibit considerable variability among strains with respect to their biocontrol activity and host range.[27] Ammonium sources of nitrogen showed maximum activity of chitinase and β-1,3-glucanase compared to other sources of nitrogen and the least activity on urea. These results were supported by the report of El-Katatny et al.,[23] where it was reported that corn steep solid was the most stimulative for chitinase production, followed by $(NH_4)_2SO_4$ or NH_4NO_3. Peptone-casein gave the least degree of enzyme activity, whereas urea gave no enzyme activity when used as a nitrogen source. Indirect support also comes from the observation that *T. viride* showed maximum biomass production on ammonium sulphate compared to sodium nitrate and potassium nitrate, whereas it showed the least growth on urea.[28]

It is widely known that environmental parameters such as abiotic (soil type, soil temperature, soil pH, water potential, and so on) and biotic factors (plant species and variety, microbial activity of the soil) as well as other factors such as method and timing of applications may have influence on the biological control efficacy of *Trichoderma* isolates. In the present study, the isolates ThJt1 and TvHt2 out of 12 *Trichoderma* isolates showed the highest enzyme activity against the *S. rolfsii* tested. These organisms can be used therefore for assessment of their synergism in biomass production and biocontrol efficacy and for their field biocontrol ability against *S. rolfsii* and *P. aphanidermatum* infecting tobacco.

Conflicts of interest

The authors declare no conflicts of interest.

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